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**Evaluation Report on the Analytical Methods submitted  
in connection with the Application for Authorisation of a  
Feed Additive according to Regulation (EC) No 1831/2003**

**Probion Forte<sup>®</sup>**  
*(FAD-2014-0038; CRL/140028)*





**Evaluation Report on the Analytical Methods submitted  
in connection with the Application for Authorisation of a  
Feed Additive according to Regulation (EC) No 1831/2003**

Dossier related to: **FAD-2014-0038 - CRL/140028**

Name of Product: ***Probion Forte®***

Active Agent (s): **Bacillus subtilis (KCCM 10941P)**  
**Bacillus coagulans (KCCM 11093P)**

Rapporteur Laboratory: **Staatliche Betriebsgesellschaft für  
Umwelt und Landwirtschaft (SMUL),  
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Date: **23/06/2016**

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Date: **24/06/2016**

## EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *Probion Forte*<sup>®</sup>, under the "category"/"functional group" 4(b), "zootechnical additives"/"gut flora stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* for laying hens, and chickens for fattening and reared for laying.

According to the Applicant, the product is intended to be marketed as a light red or brown powder containing at least  $1 \times 10^8$  Colony Forming Units (CFU)/g of *Bacillus subtilis* (KCCM 10941P), and at least  $1 \times 10^8$  CFU/g of *Bacillus coagulans* (KCCM 11093P), on a zeolite-diatomite carrier. The *feed additive* is intended to be used directly in *feedingstuffs* at a minimum/maximum content of 0.5/1.0 g/kg of *Probion Forte*<sup>®</sup> in *feedingstuffs*. Taking into account (i) the content of the microorganisms in the product and (ii) the conditions of use suggested for the product in the feed, the content of the sum of both microorganisms in *feedingstuffs* would range from  $1 \times 10^8$  to  $2 \times 10^8$  CFU/kg.

For the identification of *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P), the EURL recommends for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for genetic identification.

For the enumeration of the sum of *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P) in the *feed additive* and *feedingstuffs* the Applicant applied a spread plate procedure based on the ISO 6887-1. The EURL identified instead the ring-trial validated spread plate method EN 15784, already evaluated by EURL in the frame of previous *Bacillus spp* dossiers. Based on the performance characteristics available, the EURL recommends for official control this CEN method (EN 15784) for the enumeration of the sum of the two microorganisms in the *feed additive* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

## KEYWORDS

*Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P), zootechnical additives, gut flora stabilisers, laying hens, and chickens for fattening and reared for laying.

## 1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a feed additive or authorisation of a new use of a feed additive already authorised) for *Probion Forte*<sup>®</sup> under the "category"/"functional group" 4(b), "zootechnical additives"/"gut flora stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, authorisation is sought for the use of the *feed additive* for laying hens, and chickens for fattening and reared for laying [1][2].

According to the Applicant, the *feed additive* (*Probion Forte*<sup>®</sup>) contains as active substance two microorganisms: *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P) [2][3]. Both microorganisms are deposited at the Korean Culture Centre of Microorganisms (KCCM, Seoul, Korea) [4]. The product is intended to be marketed as a light red or brown powder containing at least  $1 \times 10^8$  Colony Forming Units (CFU)/g of *Bacillus subtilis* KCCM 10941P and at least  $1 \times 10^8$  CFU/g of *Bacillus coagulans* KCCM 11093P, on a zeolite-diatomite carrier [2][3].

The *feed additive* is intended to be used directly in *feedingstuffs* at a minimum/maximum content of 0.5/1.0 g/kg of *Probion Forte*<sup>®</sup> in *feedingstuffs* [2]. Taking into account (i) the content of the microorganisms in the product and (ii) the conditions of use suggested for the product in the feed, the content of the sum of both microorganisms in *feedingstuffs* would range from  $1 \times 10^8$  to  $2 \times 10^8$  CFU/kg.

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus subtilis* in the frame of several dossiers e.g. FADs 2009-0023; 2009-2007, 2009-0013 [5].

## 2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Probion Forte*<sup>®</sup> and their suitability to be used for official controls in the frame of the authorisation were evaluated.

### 3. EVALUATION

#### ***Identification /Characterisation of the feed additive***

##### *Qualitative and quantitative composition of impurities in the additive*

The Applicant analysed the *feed additive* for microbial contaminants (such as *Salmonella*, *Shigella*, *Staphylococcus aureus*, coliform group and moulds) by using the methods described in the technical dossier [6].

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury and aflatoxin B1) are available from the respective European Union Reference Laboratories [7].

##### *Qualitative and quantitative composition of the additive*

For identification of the strains *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P) the Applicant used morphological, cultural, physiological and biochemical characteristics (API50 CH, Api20E and Microgen Bacillus ID) as well as 16S rRNA analysis. The presented phylogenetic trees for both strains confirm the affiliation to the species *B. subtilis* and *B. coagulans* on species level [8].

Colony-morphological description of both strains where made on the basis of nutrient agar. For official control it is necessary to give a characterization on Tryptic Soy Agar for a better faculty of discrimination between native and probiotic *Bacillus spp.*

The EURL recommends instead for official control Pulsed Field Gel Electrophoresis (PFGE), a generally recognised standard methodology for microbial identification and also for description on strain level [9].

#### ***Description of the analytical methods for the determination of the active substance in feed additive and feedingstuffs***

For the enumeration of the overall (sum of) *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P) in the *feed additive* and *feedingstuffs* the Applicant applied a spread plate procedure [10] based on the ISO 6887-1 [12].

The *feed additive* sample (10g) is diluted in 90 ml of sterilised 0.85% saline solution and shaken for 1h at room temperature for dispersion, appropriately diluted and spread on the autoclaved agar medium (Tryptic soy agar with Cycloheximide 0,01%) to be cultivated for 36-48h at 37 °C for microbe before colony counting (25-250 colonies per plate). Finally the number of cells per ml is calculated by multiplying the number of colonies by the dilution factor [10].

The Applicant provided additional experimental evidence in the frame of the stability assays demonstrating the applicability of this method to enumerate the sum of all *Bacillus spp* in *feedingstuffs* for broiler chickens samples prepared in accordance to ISO 6887-1 [8][12].

The EURL identified instead for the enumeration of the sum of *Bacillus spp.* in the *feed additive* and *feedingstuffs* the ring-trial validated spread plate method EN 15784 [11]. This method was already evaluated by EURL in the frame of previous *Bacillus spp* dossiers [5].

Twenty grams of the *feed additive* (or 50g of *feedingstuffs*) are suspended in a phosphate buffered saline (PBS) (or in 0.2% sodium hydroxide solution for *feedingstuffs*). From this, one new dilution is prepared and heat-treated at 80 °C for 10 minutes. Decimal dilutions are prepared from the heat treated suspension, spread plated on tryptone soya agar (TSA) and incubated at 37 °C for 16-24 h aerobically. The following performance characteristics were reported after logarithmic transformation (CFU) [11]:

- a standard deviation for *repeatability* ( $S_r$ ) ranging from 0.07 to 0.09  $\log_{10}$  CFU/g;
- a standard deviation for *reproducibility* ( $S_R$ ) ranging from 0.32 to 0.35  $\log_{10}$  CFU/g; and
- a limit of quantification (LOQ) of  $2 \times 10^7$  CFU/kg of *feedingstuffs*.

Based on the performance characteristics available the EURL recommends for official control the ring trial validated CEN method (EN 15784) for the enumeration of the sum of *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P) in the *feed additive* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

#### 4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control (i) the CEN method EN 15784 for the enumeration of the sum of *Bacillus subtilis* (KCCM 10941P) and *Bacillus coagulans* (KCCM 11093P) in the *feed additive* and *feedingstuffs* and (ii) Pulsed Field Gel Electrophoresis (PFGE) for the identification of the strain.

##### ***Recommended text for the register entry (analytical method)***

- Identification: Pulsed Field Gel Electrophoresis (PFGE)
- Enumeration in the *feed additive* and *feedingstuffs*: spread plate method following heat treatment - EN 15784

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Probion Forte*® have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

## 6. REFERENCES

- [1] \*Application, Reference SANCO/G1: Forw. Appl. 1831/0055-2014
- [2] \*Application, Proposal for Register Entry, Annex A
- [3] \*Technical dossier, Section II, 2.2 Characteristics of the active substance
- [4] \*Technical dossier, Section II, Annex II-1\_2.3.2.4 - Certificates of the deposit
- [5] EURL Evaluation Report FADs-2009-0023; 2009-2007 & 2009-0013  
<https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0023.pdf>  
<https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0007.pdf>  
<https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0013.pdf>
- [6] \*Technical dossier, Section II, 2.6 Method of analysis and reference samples
- [7] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories
- [8] \*Supplementary Information, Section IV, Part C
- [9] European Community Project SMT4-CT98-2235. "*Methods for the Official Control of Probiotics Used as Feed Additives*", Report 20873/1 EN (2002) ISBN 92-894-6250-7
- [10] \*Technical dossier, Section II, Annex\_II\_8\_2.6.4.1
- [11] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp.
- [12] EN ISO 6887-1 – Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions

\*Refers to Dossier no: FAD-2014-0038

## 7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Betriebsgesellschaft für Umwelt und Landwirtschaft, Freistaat Sachsen, (SMUL) Nossen, Germany. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.



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## 8. ACKNOWLEDGEMENTS

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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabriels (ES)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Centre wallon de Recherches agronomiques (CRA-W), Gembloux (BE)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)